A Novel Wnt Regulatory Axis in Endometrioid Endometrial Cancer

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Abstract

The protocadherin PCDH10 is inactivated often by promoter hypermethylation in various human tumors but its possible functional role as a tumor suppressor gene is not established. In this study, we identify PCDH10 as a novel Wnt pathway regulatory element in endometrioid endometrial carcinoma (EEC). PCDH10 was downregulated in EEC tumor cells by aberrant methylation of its promoter. Restoring PCDH10 levels suppressed cell growth and triggered apoptosis in EEC cells and tumor xenografts. Gene expression profiling revealed as part of the transcriptomic changes induced by PCDH10 a reduction in levels of MALAT1, a long non-coding RNA, that mediated tumor suppression functions of PCDH10 in EEC cells. We found that MALAT1 transcription was regulated by Wnt/β-catenin signaling via TCF promoter binding and PCDH10 decreased MALAT1 by modulating this pathway. Clinically, MALAT1 expression was associated with multiple parameters in EEC patients. Taken together, our findings establish a novel PCDH10-Wnt/β-catenin-MALAT1 regulatory axis which contributes to EEC development.
Introduction

Endometrial cancer is the most common gynecologic malignancy and ranks fourth in whole malignancies among women (1). Endometrioid endometrial cancer (EEC), accounting for ~80%-90% of the whole cases, originates from epithelial cells lining the endometrium and is often associated with estrogen stimulation, hormone receptor positivity, obesity and favorable prognosis (2). Key mutational events have been characterized in EEC but the underlying molecular mechanisms involving oncogenic or tumor suppressive factors remain poorly elucidated (3,4). Recently, we have discovered a novel miR-193-YY1-APC regulatory axis that exerts functional roles in EEC development (5). The transcription factor YY1 plays an oncogenic function through epigenetic regulation of APC, a key molecule in regulating Wnt/β-Catenin signaling pathway which plays an essential role in cancer progression. In the current study, we investigated the tumor suppressive function of *PCDH10* (*Protocadherin 10*) in EEC. *PCDH10*, a member of non-clustered protocadherin subfamily, was proposed as a tumor suppressor (6-8) in multiple cancers. Inactivation of *PCDH10* due to promoter CpG hypermethylation has been detected in gastric, hepatocellular, colorectal, breast, cervical, lung, nasopharyngeal, esophageal, pancreatic and bladder cancer (6,9-16). Functional studies revealed that re-expression of *PCDH10* inhibits cell growth, reduces clonogenicity, restrains cell invasion and induces cell apoptosis, substantiating its tumor suppressor roles (10,13,14,16). Moreover, methylation of *PCDH10* manifests its significance through its association with clinical parameters. For example, in gastric cancer, methylation of *PCDH10* was identified at early stages of carcinogenesis and linked with poor prognosis (10). In colorectal cancer, *PCDH10* promoter methylation can be detected in plasma and the methylation rate in plasma is positively associated with that in tumor tissues in early-stage cancers (17). Although the involvement is extensively unraveled in a wide spectrum of cancers, the linkage between *PCDH10* and EEC is unknown and the molecular mechanisms
await exploration. Interestingly, recent studies added another layer of epigenetic control of 
*PCDH10* expression. *HOTAIR*, a long noncoding RNA (lncRNA), recruits the repressive 
chromatin-remolding complex PRC2 to *PCDH10* genomic loci, leading to the silencing of 
*PCDH10* expression (18,19). This report suggested interactions between *PCDH10* and the 
novel family of gene regulator, lncRNA.

LncRNAs are RNA species over 200 nucleotides in length and are recently discovered to constitute a large proportion of the whole transcriptome (20,21). Increasing evidences suggest the importance of lncRNAs in numerous cellular processes impacting gene regulation, often through interacting with diverse chromatin complexes (22-24). In cancer, lncRNAs are now emerging as a prominent layer of transcriptional regulation; more and more lncRNAs are found to be deregulated in different contexts, in conjunction with their tissue-specific nature, making them promising therapeutic targets (25-27). Among them, *matastasis-associated lung adenocarcinoma transcript 1* (*MALAT1*) is one of the most characterized. *MALAT1* is a ~ 7kb long, nuclear retained and ubiquitously expressed long ncRNA (28,29). Since its discovery as a prognostic factor for lung cancer metastasis (28), in the following decade *MALAT1* has been shown to be broadly up-regulated in a variety of cancer entities and to play critical roles in distinct cancer hallmark capabilities (26). For example, in liver cancer, *MALAT1* is associated with risk of tumor recurrence after liver transplantation by modulating cell viability, motility and invasiveness (30). In cervical cancer, *MALAT1* prompts cell proliferation and invasion; knockdown of *MALAT1* induces cell apoptosis (31). Additionally, in colorectal cancer, *MALAT1* harbors mutations and its processed 3’end fragment affects tumor growth and invasion (32). A myriad of *MALAT1* functions underscore its importance in tumor development and progression, however, study of *MALAT1* function in EEC is still lacking. Furthermore, mechanistic studies revealed that *MALAT1* could regulate alternative splicing, control the expression of cell cycle regulators, and modulate E2F
transcriptional activity under different cellular contexts (33-36). Notwithstanding the substantial advancements in our understanding of MALAT1 function, the transcriptional regulation of MALAT1 expression and the causes behind its deregulation in tumors are barely explored.

In the current study, we found that PCDH10 is silenced in EEC cells/tumors through its promoter CpG hypermethylation. Ectopic expression of PCDH10 inhibits tumor growth and induces cell apoptosis both in vitro in EEC cells and in vivo in a xenograft tumor. Transcriptomic analysis revealed that PCDH10 expression down-regulates MALAT1. Further mechanistic studies uncovered that MALAT1 expression is transcriptionally induced by Wnt/β-catenin signaling through a direct binding site of TCF4 in MALAT1 promoter region. Altogether our results have uncovered a novel PCDH10-Wnt/β-catenin-MALAT1 regulatory axis which contributes to EEC development and progression.
Materials and Methods

Tissue samples. EEC samples were acquired from the tissue bank of the Department of Obstetrics and Gynaecology, Prince of Wales Hospital. 76 cases of primary endometrioid endometrial adenocarcinoma (EEC) and 45 cases of normal tissues (NE) were enrolled in this study. Normal endometrial tissue specimens were obtained from women who underwent a hysterectomy or endometrial curettage for endometrial-unrelated diseases, such as uterine myoma or prolapse. Clinical staging was performed according to International Federation of Gynecology and Obstetrics (FIGO) criteria. All specimens, clinical information and procedures were approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong. Tissue microarray data of 142 endometrial cancer tumors, 19 metastatic lesions and 18 endometrial hyperplasia samples from a prospectively collected Norwegian population-based bio bank as part of MoMaTEC trial (http://www.clinicaltrials.gov/ct2/show/NCT00598845) was employed in PCDH10 and MALAT1 association analysis with clinical parameters, in accordance with approval from the Regional Committee for Medical and Health Research Ethics, Western Norway (NSD 15501). RNA was extracted from fresh frozen tumor samples with confirmed > 80% tumor purity using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and hybridized to Agilent Whole Human Genome Microarrays 44k (Cat.no. G4112F), according to the manufacturer’s instructions (www.agilent.com) and described earlier (37). Arrays were scanned using the Agilent Microarray Scanner Bundle. Raw data were imported and analyzed in J-Express software (Molmine, Bergen, Norway). Mean spot signal was used as intensity measure, and expression data were normalized using median over entire array.

Xenograft mouse model. Female athymic nude mice, aged 3-4 weeks, were used for tumor xenografts. 5 x 10^6 of empty vector control or PCDH10 stably expressing HEC-1-B cells
were subcutaneously injected into the left and right flanks of the mice (n = 5 for each group). At 13 days after implantation, the tumor became apparent. Tumor dimensions were measured every two days and the tumor volume was estimated by using the formula \( V = D \times d^2 / 2 \), where \( D \) is the long axis and \( d \) the short axis of the tumor. Mice were sacrificed 43 days post injection, and tumors were excised, weighed, and snap-frozen for RNA extraction, or paraffin-embedded for IHC staining. Si-NC or si-MALAT1 oligos were injected into the xenograft tumors for MALAT1 knockdown. Details can be found in Suppl. Information. All animal experiments were approved by CUHK Animal Experimentation Ethics Committee.

**Cell culture.** Human endometrial cancer cell lines, AN3CA, HEC-1-B, HEC-1-A, KLE, and RL95-2, were obtained from American Tissue and Cell Culture (ATCC) and cultured as recommended. To generate AN3CA or HEC-1-B cell line stably expressing PCDH10, the overexpression or vector control plasmid was transfected into the cells followed by G418 selection for 10 days. The resistant colonies were pooled together and amplified for further use. Details for viable cell counting, MTS assay, colony formation assay, wound healing assay and 5-Aza-2-Deoxycytidine (5-Aza) treatment, FACS analysis, TUNEL and luciferase reporter assay are included in Suppl. Information.

**Plasmid.** The PCDH10 expression vector was a kind gift from Prof. Jun Yu (Chinese University of Hong Kong, CUHK). Topflash reporter plasmid was a gift from Prof. Kingston Mak (CUHK). Human MALAT1 expression vector was a kind gift from Prof. Kannanganattu V Prasanth (University of Illinois). To construct the pGL3-MALAT1 wild type reporter plasmid, a 516 bp fragment harboring TCF4 binding site was amplified from HEC-1-B genomic DNA and cloned into the KpnI and NheI site of the pGL3-basic vector (Promega, Madison, WI, USA) according to manufacturer's instruction. The mutant plasmid was
generated by mutating the TCF4 binding motif from CTTCGAA to CTTCGCG using the method described previously (38). Primers used are listed in Suppl. Table S5.

**Bisulfite Genomic Sequencing of Individual Alleles.** Genomic DNA was modified by sodium bisulfite as described in Suppl. Information.

**Oligonucleotides.** siRNA against *MALAT1* or control oligos were obtained from Ribobio (Guangzhou, China). In each case, the concentration used for transient transfections was 100 nM. Sequences of siRNA oligos are listed in Suppl. Table S5.

**MALAT1 in situ hybridization.** An RNA antisense probe was *in vitro* transcribed corresponding to 6871-7224 bp of *MALAT1* (RefSeq accession, NR_002819) (29). The DNA template for this sequence was PCR amplified and sub-cloned into the *EcoRV* site of the pUC57 vector (GeneScript). The plasmid was then linearized using *BamH I* enzyme and *in vitro* transcribed to synthesize RNA probe using DIG RNA Labeling Kit (Roche Molecular Biochemicals) with T3 RNA Polymerase (Ambion) according to the manufacturer’s instructions. Details for in situ hybridization were included in Suppl. Information.

**Immunoblotting and immunostaining.** Western blotting analysis was performed according to our previous protocol (5). The following dilutions of antibodies were used: anti-β-Catenin (1:1000, 9562, Cell Signaling Technology), anti-α-Tubulin (1:5000, sc-23948, Santa Cruz), anti-Caspase-3 (1:300, sc-7148, Santa Cruz), and anti-Caspase-9 (1:2000, ab25758, Abcam). For Immunofluorescence staining of cultured cells, the following dilutions were used: anti-
Ki67 (1:200, sc-15402, Santa Cruz). For Ki67 and DAPI quantification, counting was conducted on at least 10 randomly chosen fields using Image-Pro plus6.0 software.

**ChIP-PCR assay.** ChIP assays were carried out as previously described (39). 5 μg of antibodies against β-Catenin (9562, Cell Signaling Technology) or equal amount isotype IgG (Santa Cruz Biotechnology) as a negative control was used for each 2 x10⁷ cell per ChIP. Immunoprecipitated genomic DNA was resuspended in 15 μl of water. PCR was then performed with 1 μl of DNA as a template and products were analyzed by qRT-PCR on a 7900HT system (Life Technologies). Primers for PCRs are listed in Suppl. Table S5.

**Sequencing and base calling.** Preparation of transcription libraries for sequencing on Illumina GA2x platform was carried out using mRNA-seq sample preparation kit (Part no. 1004898 Rev. D) according to manufacturer's standard protocol. Read mapping to genome with splice-aware aligner Sequenced were described in Suppl. Information.

**Statistical analysis.** Data were analyzed using SPSS 17.0 software package (SPSS, Statistical Product and Service Solution Chicago, IL, USA). The difference of PCDH10 mRNA expression between tumor and adjacent non-tumor tissues was analyzed by the Mann-Whitney U test. For tissue microarray data, the nonparametric Mann-Whitney U test was applied to assess the relationship between MALAT1 or PCDH10 expression levels and clinical parameters. For analyzing the association of MALAT1 ISH scoring with clinical parameters, Pearson's chi-square test was used. Correlation between PCDH10 and MALAT1 expression using tissue microarray data was carried out using Pearson Correlation and simple linear regression model. The difference in tumor growth rate between two groups of mice
was determined by repeated-measures analysis of variance. All quantitative data, like mRNA expression, MTS and luciferase activity data were obtained as triplicates. Data were shown as mean ± standard deviation (s.d.). Statistical significance between two groups was assessed by Student’s t-test. All tests were two sided, and $P < 0.05$ was considered statistically significant.
Results

**PCDH10 is down-regulated in EEC through promoter hypermethylation**

*PCDH10* is reported to be silenced in multiple human cancers (6,9-16). To investigate its potential involvement in EEC, we first examined its mRNA expression in five EEC cell lines, AN3CA, HEC-1-A, HEC-1-B, RL95-2, and KLE as well as micro-dissected EEC tumor samples using normal endometrial (NE) tissue as controls. Results showed that *PCDH10* was significantly down-regulated in 76 tumor samples and all five cell lines as compared with 45 normal controls (Fig 1A and Suppl. Table S1). Interestingly, no significant difference is seen between secretory versus proliferative endometrium but its level is much higher in the post-menopausal endometrium (Suppl. Fig. S1A). Next, we tested whether such down-regulation was caused by its promoter hypermethylation. In line with the previous findings from other cancer types, aberrant hypermethylation in a CpG island (+8 ~ -328 bp upstream transcriptional start site, TSS) of *PCDH10* promoter was detected in EEC tumors while not observed in NE tissues by bisulfite genomic sequencing (Fig. 1B). This finding was further strengthened by analyzing the genome-wide methylation data generated on HumanMethylation 450 array by The Cancer Genome Atlas (TCGA) project. Our analysis results from a cohort of 208 EEC patients and 34 normal controls showed a marked hypermethylation on the above region of *PCDH10* promoter in EEC samples compared with the normal controls (Fig. 1C and Suppl. Fig. 1B-C). Interestingly, mining the data we identified a second CpG island downstream of the TSS (+1194 ~ +3159) which is also significantly hypermethylated in EEC samples. Furthermore, we downloaded gene expression data generated from an Illumina GA RNA-seq platform from TCGA and explored the correlation between *PCDH10* expression levels with the methylation intensity. Expectedly, an evident anti-correlation was detected in about 40% of the samples (Fig. 1D). These samples exhibit high methylation level and a low expression; on the other hand some samples
with low methylation level exhibit either high or low expression level, resulting in a L-shape which is typical for genes repressed by methylation (40). Consistently, when treated with demethylation agent, 5-Aza, the promoter hypermethylation was markedly reduced (Fig. 1E) and PCDH10 expression was restored in EEC cells (Fig. 1F). Collectively, the above results demonstrate that PCDH10 is down-regulated in EEC cell lines and some tumor samples through its promoter hypermethylation.

**PCDH10 restoration inhibits proliferation and induces apoptosis in EEC cells**

The decrease of PCDH10 expression in EEC tumors implies that it may play a tumor-suppressive role in EEC tumorigenesis. To test this notion, we first performed gain-of-function study by overexpressing PCDH10 in two EEC cell lines, AN3CA or HEC-1-B. Successful restoration of PCDH10 expression (Fig. 2A) was found to inhibit EEC cell proliferation in a dose-dependent manner as assessed by both viable cell counting (Fig. 2B) and MTS assay (Fig. 2C). Furthermore, overexpression of PCDH10 in the two cell lines impeded their abilities to grow in an attachment-independent manner; the colonies formed in PCDH10 overexpressing cells were remarkably fewer in number and smaller in size than those in the empty vector group (Fig. 2D). PCDH10 has been found to be involved in suppression of cell migration in several cancer types (10,16). To answer whether this could be extended to EEC cells, we generated AN3CA and HEC-1-B cell lines stably expressing PCDH10 or empty vector (Suppl. Fig. S2A). Interestingly, results from wound healing assay demonstrated that PCDH10 overexpression had no detectable effect on cell motility in both cell lines (Fig. 2E).

In order to determine how cell proliferation is modulated by PCDH10, we investigated its effect on cell cycle. A significant delay in G1 and G2 progression was only observed in HEC-1-B but not in AN3CA cells (Fig. 2F). However, a marked increase in the
number of subG1 cells was detected in both AN3CA and HEC-1-B cells (Fig. 2F), suggesting that PCDH10 expression may have led to cell apoptosis. To confirm this thought, Annexin V-PI double staining revealed that overexpression of PCDH10 induced a reduction in the living cell population (Annexin V-/PI-, LL) and an accompanying increase in the early apoptotic population (Annexin V+/PI-, LR) (Fig. 2G). Consistently, TUNEL assay also showed the number of apoptotic cells was significantly increased upon PCDH10 expression (Fig. 2H); lastly, an increased level of active Caspase 3 and Caspase 9 proteins was detected in PCDH10 expressing cells (Suppl. Fig. S2B). Collectively, these results lead us to conclude that PCDH10 is a pro-apoptotic factor in EEC cells.

**RNA-sequencing reveals MALAT1 as a downstream factor of PCDH10**

To gain insights into the underlying mechanism of PCDH10 function in EEC cells, we performed a genome-wide analysis to globally characterize PCDH10-affected transcriptomic changes. PolyA+ RNAs were extracted from PCDH10-overexpressing HEC-1-B or the Vector control cells and subjected to RNA sequencing analysis. The majority of sequenced reads can be mapped to coding regions (CDS and UTRs, > 70%) and much fewer in introns, intergenic and non-coding regions (Suppl. Fig. S3A), suggesting a great specificity for expressed mRNA and rejection of genomic DNA and unspliced pre-mRNA. As a result, we found a total of 1233 and 679 genes were up- and down-regulated in PCDH10 overexpressing HEC-1-B cells with respect to control cells (Suppl. Fig. S3B and Suppl. Dataset). To validate the RNA sequencing findings, 12 genes (6 up- and 6 down-regulated genes) were randomly selected and subjected to qRT-PCR examination. The results agreed favorably with the RNA-seq data (Suppl. Fig. S3C).

Subsequent Gene Ontology (GO) analysis of the above down-regulated list of genes revealed an enrichment for GO items including “Cell cycle phase”, “Cell cycle process”,
“Mitotic cell cycle”, “M phase” and “Nuclear division” (Fig. 3A and Suppl. Dataset). Such alterations are in keeping with the cell cycle arrest and cell proliferation inhibition observed upon $PCDH10$ expression (Fig. 2). The up-regulated genes are enriched for GO categories related to “Glycoprotein”, “Cell membrane”, “Cell adhesion” etc (Fig. 3B). Although apoptosis related GO terms were not enriched, many pro-apoptotic factors, including $BAD$, $PLEKHF1$ and $FIS1$, were up-regulated (Suppl. Fig. S3D and Suppl. Dataset), supporting the observed impact of $PCDH10$ on cell apoptosis.

Among all the potential targets influenced by $PCDH10$, $MALAT1$ caught our attention because of its known oncogenic role in other cancers (26). The RNA-seq data revealed that $MALAT1$ RNA level was decreased by ~ 70% in $PCDH10$ expressing cells (Fig. 3C-D, Suppl. Dataset). To independently validate the result, qRT-PCR assay also showed that $MALAT1$ was indeed significantly down-regulated upon $PCDH10$ overexpression in both AN3CA and HEC-1-B cells (Fig. 3E).

The impact of $PCDH10$ on $MALAT1$ in expression level prompted us to ask whether $MALAT1$ is functionally downstream of $PCDH10$. To test this notion, we examined the effect of $MALAT1$ knock down in AN3CA and HEC-1-B cells by using two siRNAs oligos targeting it (siMALAT1A and siMALAT1B) and a non-targeting sequence as a control (siNC). Successful decrease of $MALAT1$ (Suppl. Fig. S4A-B) led to a significant delay in cell proliferation compared with the siNC treated cells as revealed by cell counting assay (Fig. 3F) and MTS assay (Fig. 3G). Conversely, when we overexpressed $MALAT1$ through the transient transfection of a plasmid (Fig. 3H), cell proliferation was enhanced compared to the cells transfected with an empty Vector plasmid (Fig. 3I). Furthermore, TUNEL assay showed that siMALAT1 treatment induced a striking increase in apoptotic cell population by over five folds compared with siNC treatment (Fig. 3J). Altogether the above findings suggested that $MALAT1$ knockdown phenocopied $PCDH10$ overexpression effect in EEC cells and
implicated \textit{MALAT1} as a downstream effector of \textit{PCDH10}. To strengthen this finding, we found that overexpression of \textit{MALAT1} reversed the inhibitory effect of \textit{PCDH10} on EEC cell growth (Fig. 3K).

\textit{PCDH10} suppresses \textit{MALAT1} transcription through inhibiting WNT/\(\beta\)-catenin signaling

Next we further explored the molecular mechanism underlying \textit{PCDH10} suppression of \textit{MALAT1}. Little is known about how \textit{PCDH10} down-regulation promotes tumorigenesis, but PCDH-gamma, another member of PCDH superfamily, was previously found to negatively regulate Wnt/\(\beta\)-catenin signaling (41,42). Wnt/\(\beta\)-catenin signaling pathway plays pivotal roles in programming developmental gene expression; constitutive activation of this pathway is involved in diverse cancer types (43). The abnormal activation was also found to be a common event in EEC, underscoring its clinical significance (44,45). We therefore speculated that loss of \textit{PCDH10} has contributed to the activation of Wnt signaling pathway which subsequently induces \textit{MALAT1} expression. To test this notion, we first examined the effect of \textit{PCDH10} restoration on Wnt signaling. Compared with the Vector control, enhanced expression of \textit{PCDH10} caused a sharp decrease of several known Wnt targets, including \textit{LEF1}, \textit{TCF1} and \textit{c-MYC} in both AN3CA and HEC-1-B cell (Fig. 4A). Consistently, \textit{PCDH10} overexpression repressed the activity of a Wnt signaling reporter, Topflash luciferase reporter, by \(~ 40\%\) when compared to the control (Fig. 4B). Collectively, our data implicate that \textit{PCDH10} negatively modulates canonical Wnt/\(\beta\)-catenin signaling.

Having established the connection between \textit{PCDH10} and Wnt/\(\beta\)-catenin signaling, we further asked whether \textit{PCDH10} influences \textit{MALAT1} expression through this pathway. Given that Wnt/\(\beta\)-catenin signaling transcriptionally activates the expression of a myriad of genes, we evaluated its effects on \textit{MALAT1} transcription. Activation of Wnt signaling by treatment
of the EEC cells with lithium chloride (LiCl) led to a mild but significant increase of MALAT1 expression as well as other known targets, c-MYC, TCF1 or LEF1 (Fig. 4C), suggesting Wnt activation indeed induces MALAT1 transcription.

To further ask whether the regulation is directly through TCF/\(\beta\)-Catenin binding to MALAT1 promoter, we searched for TCF binding sites by analyzing publically available TCF4 ChIP-seq data generated from various cell lines (HeLa-S3, MCF-7, HCT-116, HEK293 and PANC-1) (46) by ENCODE. In all the five cell lines, two TCF4 binding peaks proximal to the TSS of MALAT1 were detected (Fig 4D, Suppl. Fig S4C), suggesting a possibility for the direct binding of TCF4 on MALAT1 promoter. Indeed, a consensus binding motif of TCF4 was found in the promoter region (+78 to +88 bp) using rVISTA 2.0 tools (47) (Fig. 4D, Suppl. Fig. S4C). To experimentally test TCF association with this site, we cloned the genomic region harboring it into a luciferase reporter (wild type, WT) and transfected it into EEC cell to test its response to Wnt activation. Results showed that the reporter activity was induced upon Wnt activation by LiCl treatment in a dose dependent manner; however, the response was lost when we mutated the TCF binding site by altering the consensus sequence (Mut) (Fig. 4E). These results suggested that Wnt signaling probably activates MALAT1 transcription through the identified TCF4 binding site. Furthermore, when examining the effect of PCDH10 on the reporter activity, we found PCDH10 over-expression suppressed the WT but not the Mut reporter activities (Fig. 4F), suggesting PCDH10 regulates MALAT1 transcription through inhibiting Wnt signaling. To strengthen the above findings, we performed chromatin immunoprecipitation (ChIP) to detect \(\beta\)-catenin enrichment on MALAT1 promoter. Expectedly, as shown in Fig. 4G, a specific and robust enrichment of \(\beta\)-catenin (~ 8 fold) was found on the identified TCF4 site, which is comparable to its enrichment on a known target, c-Myc; and the enrichment was significantly diminished (~ 4 fold) upon PCDH10 overexpression.
Together, these data demonstrate that MALAT1 genomic locus is under the direct transcriptional regulation of Wnt/β-catenin signaling and PCDH10 suppresses MALAT1 expression through impairing β-catenin binding to its promoter. To further answer the question of how PCDH10 affects β-catenin binding, we tested whether it has any impact on β-catenin expression or its nuclear translocation. The results on the other hand indicated that over-expression of PCDH10 had no impact on the total β-catenin expression and however caused slight decrease of nuclear β-catenin level (Suppl. Fig. S5).

**PCDH10-MALAT1 regulatory axis in vivo**

Lastly, we evaluated the function of PCDH10-MALAT1 regulatory axis in vivo firstly using a xenograft tumor model established by injecting HEC-1-B cells stably expressing PCDH10 or empty vector into nude mice. Measuring the tumor size every 3 days, we found PCDH10 overexpression markedly delayed tumor growth from the very beginning when compared with Vector control (n = 5; P < 0.01) (Fig. 5A). Tumor size and mass were evidently smaller at the end of evaluation (Fig. 5B and C) when PCDH10 over-expression was also confirmed by qRT-PCR (Fig. 5D). TUNEL assay on the paraffin sections revealed that PCDH10 overexpression caused a severe cell apoptosis in vivo (Fig. 5E), which was in agreement with its pro-apoptotic effect found in vitro. In order to illustrate the effect of MALAT1, siRNA oligos were injected into the HEC-1-B xenograft tumor to knockdown MALAT1 in vivo. A significant delay in tumor growth was observed during a 10- day measurement course (n = 5; P < 0.01) (Fig. 5F). Tumor size and mass were also decreased in the end stage tumors (Fig. 5G-I). These results demonstrated MALAT1 decrease led to a comparable phenotype as PCDH10 over-expression. Consistently, we also detected a reduced level of both MALAT1 and c-Myc expression in PCDH10 overexpressing tumors (Fig. 5J), suggesting the existence of PCDH10-Wnt-MALAT1 axis in vivo in the xenograft tumors.
Next, we validated the above findings in EEC clinical samples. First, we examined the \( \text{MALAT1} \) expression in 32 EEC samples by in situ hybridization on the paraffin sections using a \( \text{MALAT1} \) specific probe (29). As expected, \( \text{MALAT1} \) signal was mainly detected in the nuclei of endothelial cells and a much higher level was found in EEC samples as compared with the 9 normal tissues (Fig. 5K and Suppl. Table S2). In addition, a strong \( \text{MALAT1} \) signaling appeared to be associated with low histologic grade (grade 1/2 versus 3) (\( P = 0.028 \)) (Fig. 5L); However, we did not observe an association between \( \text{MALAT1} \) level and FIGO stage (Suppl. Fig. S6A). Like \( \text{PCDH10} \), \( \text{MALAT1} \) expression appears to be regulated during the menstrual cycle; it is higher in proliferative compared with secretory and post-menopausal samples (Suppl. Fig. S6B). To further evaluate potential association of \( \text{MALAT1} \) with other clinical parameters, we explored tissue microarray data performed on an independent validation set of 142 EEC and 18 hyperplasia samples (45) (Suppl. Table S3). The results revealed that high expression of \( \text{MALAT1} \) was linked with hyperplasia (\( P = 0.017 \)), menopausal status (\( P = 0.028 \)), no recurrence (\( P = 0.032 \)) and low metastasis potential (\( P = 0.041 \)) (Table 1). \( \text{PCDH10} \) on the other hand only exhibited association with hyperplasia (\( P = 0.013 \)) (Suppl. Table S4). Further exploring the TAGA gene expression data from a large cohort of EEC samples (\( n = 253 \)), we also detected a reverse association between \( \text{PCDH10} \) and \( \text{MALAT1} \) expression levels, although with a statistical significance (\( P = 0.0324 \)) (Fig. 6M). This anti-correlation was however not found from analyzing the tissue microarray data probably due to its smaller sample size. Additionally, to further validate the regulation between Wnt/\( \beta \)-catenin and \( \text{MALAT1} \), we stained \( \beta \)-catenin by immunohistochemistry (IHC) on a subset (\( n = 37 \)) of EEC tumors. The expression level of \( \text{MALAT1} \) by ISH staining is found strongly correlated with the total or nuclear level of \( \beta \)-catenin by IHC staining (Fig. 6N and O). However, no correlation was detected between \( \text{PCDH10} \) expression and \( \beta \)-catenin.
signal (total or nuclear level) (data not shown). Altogether, these analyses confirm the presence of PCDH10-Wnt/β-catenin-MALAT1 regulation in clinical samples.
Discussion

In the current study, we identified *PCDH10* as a tumor suppressor down-regulated in EEC. Although the down-regulation of *PCDH10* has been reported in a wide range of cancers (6,9-16), as far as we know, this is the first study to demonstrate its association with EEC. Our results clearly showed that *PCDH10* promoter is hypermethylated through both genomic bisulfite sequencing analysis of locally collected EC samples and analyzing TCGA data from worldwide EEC samples. Thus, loss of *PCDH10* expression through its promoter hypermethylation appears to be a common event occurring in many tumors. Exploiting the TCGA datasets, ours is the first to report an anti-correlation between *PCDH10* promoter CpG methylation levels with its mRNA expression in ~40% of EEC tumors examined, thus providing solid evidence to support that the hypermethylation causes *PCDH10* silencing in these tumors. This type of methylation-expression correlation analysis has not been reported in any other *PCDH10* studies. Notably, in addition to the previously known promoter CpG island, we also identified a second CpG island downstream of TSS that is hypermethylated in EECs; it will be interesting to explore whether it also contributes to *PCDH10* silencing in the future. Consistently, recent integrative genomic analysis of endometrial cancers did not reveal copy number alterations or mutations on *PCDH10* gene, reinforcing the significance of epigenetic down-regulation (3). Interestingly, in addition to promoter hypermethylation, recent studies uncovered alternative epigenetic events partaking in its down-regulation in breast cancer and pancreatic cancer (18,19). In these reports, PRC2 complex were found to be recruited, mediated by *HOTAIR*, to bind the promoter region of *PCDH10*, resulting in an increase in repressive chromatin mark, H3K27me3. Such cooperative action of DNA methylation and histone modifications in orchestrating target gene expression has been extensively revealed in developmental and pathological processes, including cancer (48). For example, in our recent work, we demonstrated APC promoter, in addition to under regulation
of DNA methylation, is subjected to YY1 mediated EZH2 recruitment and subsequent H3K27me3 modification, leading to its inactivation (5). Indeed, we also noticed a substantial subset of EEC samples (n=58) with low PCDH10 expression do not bear promoter hypermethylation when analyzing TCGA data (Fig. 1C and D), suggesting additional mechanisms may exist to repress PCDH10 expression in these samples.

Further functional studies revealed PCDH10 could be a potential modulator of EEC carcinogenesis both in vitro and in vivo. Restoration of PCDH10 successfully reduced cell growth and induced cell apoptosis, which is in keeping with what was uncovered in gastric, lung and esophageal cancers. Notably, we could not recapitulate all functional features of PCDH10 demonstrated in other studies (10,13,14,16). For example, in gastric cancer cells, PCDH10 was shown to exert significant impacts on cell migration (10), however, this was not observed in the two EEC cell lines investigated in our study. The impact of PCDH10 on cell cycle also differs between two EEC cell lines (Fig. 2F). These discrepancies may reflect cell type and cancer type specific roles of PCDH10.

Despite the considerable advancements in our understanding of the tumor suppressive functions of PCDH10 in distinct cancer types, nothing is known about the underlying molecular basis. Our findings, for the first time, uncovered the transcriptomic influence exerted by PCDH10 through RNA-seq. Moreover, we identified MALAT1 as a functional downstream target of PCDH10. MALAT1, as one of the most characterized lncRNAs, has been broadly associated with cancer development and engaged in diverse facets of cancer progression. Our report, nonetheless, represents the first demonstrating its functionality in EEC. In keeping with previous reports, MALAT1 plays an oncogenic role in EEC cells and tumors. Additionally, we demonstrated that MALAT1 is positively associated with hyperplasia, and negatively with metastasis, suggesting its predictive value as a molecular biomarker. Of note, the reverse association between MALAT1 and the metastasis status in
EEC is distinct to the findings from other cancer types. In lung cancer, high expression of *MALAT1* is linked with metastasis and *MALAT1* regarded as a critical regulator of metastasis phenotype (28,36). In bladder cancer, high *MALAT1* expression level is also connected with high-grade and high-stage carcinoma (49). These findings suggest that *MALAT1* function is cancer-type dependent. Linkage between high *MALAT1* expression with hyperplasia, to some extent, reflects its roles in cell proliferation. Endometrial hyperplasia is the result of excessive proliferation of the endometrium cells and is considered as a remarkable risk factor for the development of EEC or even coexists with EEC (45). The positive correlation of *MALAT1* with hyperplasia and low grade EEC implies that dysregulation of *MALAT1* is an early event in EEC development. The lower *MALAT1* expressions in metastasis and tumors leading to recurrences or progressions point to other carcinogenic mechanisms as prevailing in the late stages of development as well as the most aggressive endometrial cancers.

In this study, we also provide novel insights into how *PCDH10* acts on *MALAT1* through modulating Wnt/β-catenin signaling. We identified *MALAT1* as a direct transcriptional target of Wnt/β-catenin in EEC cells. A TCF4 binding site was identified on its promoter region which was proven to mediate Wnt effect on *MALAT1*. According to ChIP-seq data from ENCODE, the TCF binding on this site is present in multiple cancer cell lines including HeLa-S3, MCF7, HCT116 and PANC-1, thus Wnt regulation of *MALAT1* likely occurs as a general pathway in various cancers. In addition to TCF binding, in SK-N-SH cell, cyclic AMP-responsive element binding (CREB) transcription factor was reported to be associated with *MALAT1* promoter region (50). Recent study revealed *MALAT1* is also under the regulation of one miRNA, miR-9 (51). Considering its length and high abundance in many cell entities, we speculate that *MALAT1* is under tight control of many factors. Indeed, ChIP-seq and Chromatin State Segmentation data from Encode project revealed binding peaks of many transcription factors and histone marks upstream its promoter (29,46).
Aberrant activation of Wnt/β-catenin signaling pathway occurs commonly in EEC and presumably occurs early leading to EEC initiation (44). It is often caused by mutations in β-catenin or Wnt antagonists (e.g. APC), resulting in nuclear accumulation of β-catenin and augmented transcription of downstream targets (4). However, enhanced Wnt/β-catenin signaling is also observed in EEC tumors which do not carry such mutations, suggesting alternative mechanism underlying Wnt activation. Recently, protocadherin family members were implicated in crosstalks with the canonical Wnt/β-catenin signaling pathway. In Wilms’ tumor (nephroblastoma), PCDH gamma members are able to repress β-catenin/TCF mediated transcription and suppress β-catenin/TCF reporter activity (42), which is also supported by evidence from colon cancer (41). In line with these findings, our current study also uncovered a link between PCDH10 and Wnt signaling, suggesting an alternative means through PCDH10 silencing to activate Wnt/β-catenin signaling. Exactly how PCDH10 suppresses Wnt/β-catenin signaling activity is still unclear. From our results (Suppl. Fig. S5), PCDH10 re-expression did not affect the total cellular β-catenin level and caused slight decrease of nuclear β-catenin level, suggesting PCDH10 effect on β-Catenin transcriptional activity may not be directly through regulating its expression or nuclear translocation; this was also supported by the findings from EEC samples where no significant correlation between β-catenin (total or nuclear) and PCDH10 levels was observed. Thus, PCDH10 effect on Wnt/β-catenin signaling may be mediated through other factors in this pathway such as TCF or LEF; these questions warrant future investigation on this aspect.

Collectively, we have identified a novel regulatory axis, PCDH10-Wnt-MALAT1 in the effort of elucidating the tumor suppressive function of PCDH10 (Fig. 6), rendering this the first report to show PCDH10 and lncRNA function in EEC and to elucidate the transcriptional regulation of MALAT1. It will be of great interest to explore the clinical significance of PCDH10 and MALAT1 in the future.
Acknowledgements

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References


Table 1 MALAT1 RNA expression from 142 prospectively collected endometrial cancer tumors, 19 metastatic lesions and 18 endometrial hyperplasia samples in relation to clinicopathologic data

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<th>Variable</th>
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*Mann-Whitney U-test, †Information missing for one case, ‡Lymph node sampling performed for 100 patients
Figure legends

**Figure 1. **PCDH10 is down-regulated in EEC cells through promoter hypermethylation. (A) Expression of PCDH10 in microdissected normal endometrial tissues (NE, n=45), EEC primary tumors (EEC, n=76), and five EEC cell lines. Data are plotted as Mean ± SEM. (B) Methylation status of CpG sites in the PCDH10 promoter in 8 NE and 8 EEC samples assayed by bisulfate genomic sequencing (BGS). A 336-bp region (-328 ~ +8) spanning the core promoter harboring 27 CpG sites was analyzed. Each CpG site is indicated as a short vertical bar at the top row. The degree of methylation was measured as percentage of methylated cytosines from 7 randomly sequenced colonies. (C) The scatterplot compares the methylation intensity of the above CpG region in 208 EEC tumor samples (red circles) versus normal samples (green circles) from TCGA. Y-axis: methylation intensity measured in β-value (the ratio of the methylated probe intensity and the overall intensity (sum of methylated and unmethylated probe intensities). The P-value was calculated by Student’s t-test. (D) The scatterplot compares the mRNA expression (y axis) versus DNA methylation (x axis) in 196 EEC tumors from TCGA. Each red dot is an EEC tumor sample, whereas each green dot is one of the nine normal samples. The PCDH10 promoter is silenced in the majority of these tumors, either by hypermethylation (high DNA methylation and low mRNA expression) or an unknown alternative mechanism. The mRNA expression data were obtained from IlluminaGA RNA-seqV2 platform and interpreted as log ratios. (E) Methylation status of CpG sites in the PCDH10 promoter in 5 EEC cell lines treated without (control) or with 5’-Aza. (F) Relative PCDH10 expression levels in five EEC cell lines treated with 5’-Aza for the indicated times. **P < 0.01, ***P < 0.001.

**Figure 2. **PCDH10 inhibits EEC cell proliferation and induces cell apoptosis. (A) Ectopic expression of PCDH10 in AN3CA cells (Top) and HEC-1-B cells (Bottom) by transfecting a
PCDH10 expressing plasmid at different doses (1.0 or 2.0 ug) or an empty vector control (0 ug). (B-C) Cell proliferation in the above transfected cells was measured by counting viable cell numbers or MTS assay. The data are plotted as mean ± SD from three independent experiments. (D) Left: Monolayer colony formation assay was performed in the above control or PCDH10 expressing cells. Right: The number of colonies was counted from three independent experiments. Data are plotted as mean ± SD. (E) Left: Wound healing assay was performed in the above cells and phase-contrast pictures of the wound were taken at indicated time points. Right: The percentage of wound closure was quantified at each indicated time point. Data are plotted as mean ± SD from three independent experiments. (F) Relative cell numbers in each cell cycle phase were determined by FACS of the PI stained staining Vector control or PCDH10 expressing cells. Percentages of cells in each phase (G1, S, G2, subG1) are represented and data are from three independent experiments, plotted as mean ± SD. (G) Cell apoptosis was determined by Annexin V/PI double staining of the above cells. A representative data is shown (Left). Percentages of cells in each phase (LL, viable; LR, early apoptotic; UL and UR, late apoptotic/necrotic cell) are calculated from three independent experiments (Right). Data are plotted as mean ± SD. (H) TUNEL assay was performed and the representative images are shown (Left). The index of TUNEL-positive cells is represented as the ratio of TUNEL stained cells to the total number of cells stained with DAPI (Right). A minimal of 15 fields were counted from five sections at 200× magnification. Scale bar = 50 μm. Data are plotted as mean ± SD. *P < 0.05, **P < 0.01, ****P < 0.0001

Figure 3. Genome-wide analysis by RNA-seq reveals MALAT1 as a downstream mediator of PCDH10. (A-B) GO analysis of genes that were down- or up-regulated in PCDH10-overexpressing cells. The y axis shows GO terms and the x axis shows statistical significance (i.e., P-value) for the top 10 enriched terms. (C) Differential expression of MALAT1 transcripts determined by RNA-seq, shown as wiggle tracks on the UCSC genome
browser. (D) The expression values of MALAT1 in FPKM. (E) MALAT1 RNA expression was measured in AN3CA and HEC-1-B cells expressing Vector or PCDH10 using qRT-PCR. (F-G) Depletion of MALAT1 by siRNA oligos in AN3CA (Top) and HEC-1-B cells (Bottom). Two siRNAs targeting MALAT1 (siMALAT1A and siMALAT1B) were used with a scramble sequence as control (siNC). Proliferation of the above transfected cells was determined by cell counting or MTS assay. (H) Ectopic expression of MALAT1 in AN3CA (Top) and HEC-1-B cells (Bottom) by transfecting an MALAT1 expressing (MALAT1) or a Vector control plasmid (Vector). (I) Measurement of cell proliferation in the above transfected cells by MTS assay. (J) Cell apoptosis was determined by TUNEL assay in AN3CA (Top) and HEC-1-B cells (Bottom) transfected with siMALAT1 or siNC. Representative figures are shown (Left). The index of TUNEL-positive cells is calculated (Right). Scale bar = 50 μm. (K) HEC-1-B cells stably expressing PCDH10 or control Vector were transiently transfected with MALAT1 expressing or a vector control plasmid, respectively. Cell proliferation was measured using MTS value at the indicated days after seeding. **P < 0.01, ***P < 0.001, n.s., no significance

**Figure 4. PCDH10 inhibits MALAT1 transcription through Wnt/β-catenin signaling pathway.** (A) PCDH10 decreases the mRNA expression levels of c-Myc, LEF1 and TCF1, in both AN3CA and HEC-1-B cells. (B) Transient expression of PCDH10 inhibits TOP-flash luciferase reporter activity in the above cells. (C) Expression of MALAT1 was increased by lithium chloride (LiCl) treatment; c-Myc, LEF1 or TCF1 expression was used as positive controls. (D) Schematic illustration of the promoter region of MALAT1 gene. The predicted TCF4 binding site with genomic location (+78 ~ +88) was displayed; WT, Mutant and the consensus TCF binding sequences were indicated below. (E) LiCl treatment increased the activity of the WT but not the Mut reporter. (F) PCDH10 inhibits the WT but not the Mut
reporter. Values were normalized by renilla levels and are plotted as Mean ± SD. (G) Left: ChIP-PCR detection of the β-catenin enrichment on the TCF binding site in HEC-1-B cells stably expressing PCDH10 or Vector control. Right: c-Myc genomic region harboring a TCF binding site was used as a positive control. Enrichment values are relative to input and presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01. ***P < 0.01; n.s., no significance.

Figure 5. PCDH10-MALAT1 regulatory axis in vivo. (A) PCDH10 attenuates subcutaneous tumor growth in a mouse xenograft model. Relative tumor volumes are shown with respect to day 0 where the volumes were set to 1. (B-C) Mice were sacrificed at the end of the treatment and images were taken along with the dissected tumors from three representative mice; Tumor mass was measured. (D) The over-expression of PCDH10 mRNA in the above tumors was confirmed by qRT-PCR. (E) In situ cell apoptosis in xenograft tumors was determined by TUNEL staining of the tumor sections. Scale bar = 50 μm. (F) Knockdown of MALAT1 by intratumoral injection of siRNA oligos inhibits subcutaneous tumor growth in a mouse xenograft model. (G-H) Images of mice and the dissected tumors were taken at the end of the treatment and tumor mass was measured in three represented mice. (I) The decrease of MALAT1 RNA in the above tumors was confirmed by qRT-PCR. (J) The expression of MALAT1 and c-Myc was decreased in PCDH10 xenograft tumors. Data from three representative mice were shown. (K) In situ hybridization (ISH) detection of MALAT1 RNA in normal endometrial tissues (NE) and EEC patient samples. Representative images with various levels of staining (negative from normal tissue, weak, moderate or strong from tumor tissues) were shown at 100x magnification. Scale bar = 100 μm. (L) The association of the ISH staining scores with grades of tumor (1/2 or 3). (M) A reverse correlation of PCDH10 and MALAT1 expression was examined in 253 EEC specimens from TCGA database. The correlation was determined
by Pearson's correlation test (r = 0.134; p = 0.0324). (N) IHC staining of β-catenin and ISH staining of MALAT1 on sequential sections of 37 EEC specimens. Representative β-catenin and MALAT1 staining images are shown in three EEC cases. Scale bar = 200 μm. (O) MALAT1 and nuclear β-catenin staining levels above were scored and the anti-correlation between MALAT1 ISH score and nuclear β-catenin IHC score in 37 EEC samples was shown. *P <0.05; **P <0.01.

**Figure 6. A model of PCDH10-Wnt/β-catenin-MALAT1 axis in EEC development.** In EEC tumors, the promoter region of PCDH10 is highly methylated (●) resulting in the down-regulation of PCDH10 (↓), which subsequently induces the expression of MALAT1 (↑) through activating Wnt/β-catenin signaling. The expression of MALAT1 leads to increased cell proliferation which contributes to EEC development.
Figure 1

A

B

C

D

E

F

PCDH10/CARDH

NE (n=45)  EEC (n=76)  Cell lines (n=5)

-328

+8

TSS

NE690  NE647  NE341  NE626  NE648  NE653  NE660  NE642  U410  U347  U326  U340  U411  U418  U462

<10%  10-25%  26-50%  51-80%  >80%

Methylation intensity

Tumor (n=208)  Normal (n=34)

p=1.96×10^{-14}

mRNA expression (log2 Exp)

Tumor (n=198)  Normal (n=9)

Control

AN3CA  HEC-1-B  HEC-1-A  RL95-2  KLE

5-Aza

AN3CA  HEC-1-B  HEC-1-A  RL95-2  KLE

Expression fold

5-Aza (hrs)  0  72  144
Figure 4

A

\begin{align*}
\text{AN3CA} & \\
\text{HEC-1-B} & \\
\text{Vector} & \text{PCDH10} & \\
\end{align*}

B

\begin{align*}
\text{AN3CA} & \\
\text{HEC-1-B} & \\
\text{Vector} & \text{PCDH10} & \\
\end{align*}

C

\begin{align*}
\text{AN3CA} & \\
\text{HEC-1-B} & \\
\text{NaCl} & \text{LiCl} & \\
\end{align*}

D

\begin{align*}
\text{TSS} & \text{+78++88 (TCF4)} \\
\text{CTCTTTGAAA} & \text{Wild type (WT)} \\
\text{CTCTTTGCGA} & \text{Mutant (Mut)} \\
\text{CTTGG(A/T)(A/T)} & \text{Consensus TCF4 binding site} \\
\end{align*}

E

\begin{align*}
\text{MALAT1} & \\
\text{Vector} & \text{PCDH10} & \\
\text{WT} & \text{Mut} & \\
\text{IgG} & \text{β-catenin} & \\
\end{align*}
Figure 6
A Novel Wnt Regulatory Axis in Endometrioid Endometrial Cancer

Yu Zhao, Yihua Yang, Jone Trovik, et al.

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